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ORIGINAL ARTICLE

pH-optima in lipase-catalysed esterification

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Abstract

Though lipases are frequently applied in ester synthesis, fundamental information on optimal pH or substrate concentration, can almost only be found for the reverse reaction – hydrolysis. This study demonstrates that the pH-optima of lipase-catalysed esterifications differ significantly from the optima of the hydrolysis reaction. In the esterification of *n*-butanol and propionic acid with lipases of *Candida rugosa* (CRL) and *Thermomyces lanuginosa* (TLL) pH-optima of 3.5 and 4.25, respectively, were found. This is about 3–4 units (CRL) and 7 units (TLL) in pH lower than optimum for hydrolysis. Enzyme activity increased with increasing concentrations of protonated acid indicating that the protonated acid rather than the deprotonated form is the substrate for esterification. The rate of esterification can be drastically increased by ensuring acid concentrations up to 1000 mmol L^{-1} for CRL and 600 mmol L^{-1} for TLL in the reaction system.

Keywords: *Esterification, Lipase, pH-optimum*

Introduction

Due to their ability to catalyse the hydrolysis, transesterification and synthesis of esters, combined with an excellent stability resulting in suitable catalysts for various non-conventional media (Borzeix et al. 1992; Lamare et al. 2001; Schöfer et al. 2001; Garcia et al. 2004), lipases are among the most frequently employed enzymes in industrial biotechnology (Saxena et al. 1999; Jaeger & Eggert 2002; Straathof et al. 2002).

Associated with this application in production processes, lipases and their underlying reaction mechanisms have been investigated for many years. This includes studies on the interfacial activation of lipases during hydrolytic reactions (Sarda & Desnuelle 1958) and on the 3-D structure describing the active site and the general pattern of the α/β-hydrolase fold (Brady et al. 1990; Jager et al. 1992; Ollis et al. 1992). In addition, for hydrolytic reactions, the pH activity profiles for many lipases have been characterised (Saxena et al. 1999; Sharma et al. 2001), and were recently supported by findings on the molecular level (Neves-Petersen et al. 2001).

In contrast to this extensive knowledge on lipase-mediated hydrolytic reactions, little solid mechanistic information is available on lipase-catalysed esterification reactions. The vast majority of papers published on lipase-catalysed esterification focus on technical use, process development and optimisation (e.g. Linder et al. 2005), although a fundamental analysis of mechanistic principles occurring with esterification reactions is not available.

The main reason for this lack of fundamental knowledge is probably the intrinsic problems associated with the experimental assessment of systems used for lipase-catalysed esterification. For thermodynamic reasons, such esterifications are typically carried out in organic solvents or in biphasic aqueous–organic systems, as in purely aqueous environments hardly any product formation is achieved (Kvittingen 1994). In organic solvents,

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however, water produced during the esterification reaction often forms a separate aqueous phase around the dissolved enzyme particle, caused by the low solubility of water in the typically hydrophobic organic solvents (Krishna & Karanth 2002). Besides measuring the reactant concentrations in the organic phase, such a multiphase system, being formed during the course of the reaction, makes experimental analysis difficult. The acquisition of data from the aqueous microenvironment around the enzyme is hampered due to the fact that the aqueous phase is not directly accessible e.g. to conventional instrumental pH measurement or to sample withdrawal. Information such as substrate and product concentration as well as pH present in the ultimate vicinity of the enzyme, however, would be required for an analysis of the enzyme reaction mechanism.

The difficulties outlined account for the few attempts that have been undertaken to acquire data from the water phase around enzymes, when these were used in organic solvents. Cambou and Klibanov (1984), for example, used conventional pH indicators, which partition between the aqueous and the organic phase, to visualise the pH in a system with a trapped water phase. Due to the inaccuracy of this method, the group of Halling developed water insoluble pH indicators to access the pH in a trapped aqueous phase (Brown et al. 1990; Valivety et al. 1990). The hydrophobic indicator employed remained in the organic phase and responded to the pH of the adjacent aqueous phase. These authors found that the strong partitioning of polar acids into the aqueous phase shifts its pH to lower values. They concluded that this might consequently influence the enzymatic activity in a negative manner (Cambou & Klibanov 1984; Valivety et al. 1990; Brown et al. 1990; Halling 1994; Partridge et al. 2000).

Until now, pH-optima of lipase-catalysed esterification reactions have not been reported. Most commonly, it is tacitly assumed that lipases show a similar pH optimum for esterifications as for hydrolytic reactions, which commonly lies in the neutral to alkaline range (González-Navarro & Braco, 1997, 1998; Tweddell et al. 1998; Dosanjh & Kaur 2002; Miyako et al. 2003). Whether this assumption is justified, however, is unclear. In addition, previous investigations did not evaluate whether lipases use the protonated or the deprotonated acid as substrate.

Since such fundamental knowledge is unavailable for lipases, but extremely important for rational process development, this study addresses pH-optima and the dissociation state in which the acid is used as a substrate in lipase-catalysed esterifications by a systematic and in-depth analysis. As representative catalysts, the frequently applied lipases of *Candida rugosa* (CRL) and *Thermomyces lanuginosa* (TLL) were used for the esterification of *n*-butanol and propionic acid as a model reaction. The pH-optima of these lipases for the hydrolysis of tributyrin were shown to be quite different (Neves-Petersen 2001). Therefore, it was anticipated that the pH-optima for esterification could differ in a similar manner.

Material and methods

Materials

The lyophilised lipase of *Thermomyces lanuginosa* (TLL; Chirazyme L-8) was obtained from Roche Diagnostics (Mannheim, Germany), whereas the lyophilised lipase of *Candida rugosa* (CRL) was purchased from Sigma-Aldrich (Taufkirchen, Germany). Both enzymes were used without further purification. Butyl propionate standard was from Merck-Schuchhardt (Hohenbrunn, Germany). Propionic acid, *n*-butanol, *n*-hexane, and other chemicals, all reagent grade, were obtained from Fluka (Taufkirchen, Germany).

Experimental setup

Investigations were carried out in an aqueous/organic biphasic reaction system. The setup is illustrated in Figure 1. The volumetric ratio of hexane and water was 1 and the total liquid volume in a 25 mL sealable glass reaction vessel was 14 mL. The two phases were agitated by magnetically driven paddles connected by a shaft (cf. Figure 1) with a

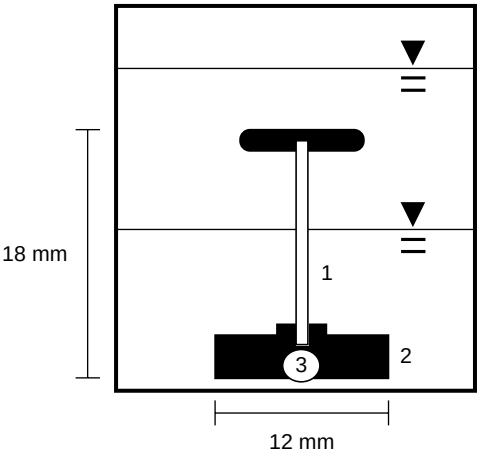

Figure 1. Experimental setup of the biphasic system – upper phase: 7 mL *n*-hexane with 150 mmol L^{-1}; lower phase: 7 mL aqueous phase with lipase and various amounts of propionic acid; each phase is stirred by magnetic driven paddles connected by a shaft in order to avoid a concentration gradient in each phase (1: stainless steel; 2: POM (polyoxymethylene); 3: magnetic stir bar).

stirring speed of 250 rpm. Higher velocities were not applicable without disturbance of the interface.

Experimental procedure

The pH activity profile of both lipases was determined for three different concentrations of propionic acid (75 mmol L^{-1}, 150 mmol L^{-1}, 300 mmol L^{-1}). Each lipase was solubilised in the presence of propionic acid in deionised water (15 mg L^{-1} CRL; 0.2 mg L^{-1} TLL). These solutions were adjusted to the desired pH (2; 2.75; 3.5; 4.25; 5; 6.25; 7.5) using either 10.2 mol L^{-1} HCl or 7.5 mol L^{-1} NaOH. In order to start the reaction, 7 mL of the aqueous propionic acid/enzyme solution were transferred into the reaction vessel followed by 7 mL of *n*-hexane containing 150 mmol L^{-1} *n*-butanol and 20 mmol L^{-1} *n*-decane as an internal standard for GC analysis. At six time points 75 μL of each phase were withdrawn in order to maintain the volumetric ratio, but only the organic phase was analysed by GC/FID (isothermal at 130°C; detector temperature at 220°C; carrier gas N_2 at 1 mL min^{-1}; column: CS-Cyclodex, length 25 m, inner diameter 0.5 mm, film thickness 0.25 μm; CS-Chromatographie, Langerwehe, Germany). The reaction was stopped after 8 h and the previously adjusted pH was checked again. Experiments were carried out at least in duplicate. For evaluation of substrate kinetics for propionic acid, concentrations up to 2500 mmol L^{-1} (TLL) and 3000 mmol L^{-1} (CRL) were applied at a pH of 3.5 for CRL and 4.25 for TLL, while all other experimental parameters were the same as mentioned above.

For investigation of propionic acid mass transfer between the aqueous and organic phase, 300 mmol L^{-1} were dissolved in deionised water and the pH adjusted to 2.75 and 5.0. The experiment was started by transferring 7 mL propionic acid solution into the reaction vessel and adding 7 mL of *n*-hexane containing 20 mmol L^{-1} *n*-decane (internal standard). For investigation of the mass transfer of butanol, 150 mmol L^{-1} *n*-butanol and 20 mmol L^{-1} *n*-decane were dissolved in *n*-hexane. The aqueous phase was free of enzyme and acid and the pH was also adjusted to 2.75 or 5.0, respectively. For these experiments, sampling and analysis were done as described before.

Results and discussion

Characterisation of the investigation system and design of experiments

When investigating enzyme-catalysed reactions where acidic compounds act as substrate, pH-optima cannot be determined according to common protocols, i.e. by measuring enzyme activity at different pH values but constant initial concentration of substrates. This results from the pH-dependency of the acid's protonation state, which changes the ratio of protonated and deprotonated species at a constant total acid concentration. The two acid species cannot be considered equal because the different charge of the protonated and deprotonated species allows for different interaction with the catalytic site of the enzyme. Thus, enzyme activity determined at constant total acid concentration would reveal a non-justified pH-dependency.

In microaqueous or biphasic systems typically employed for lipase-mediated esterifications, the acid substrate is normally provided via the organic phase. The acid protonation/deprotonation in the aqueous phase as well as the overall concentration of the acid in the aqueous phase depend on the pH. This latter dependency is due to the different partition coefficients of both acid species: while the deprotonated acid can only be detected in the aqueous phase, the protonated acid also partitions into the organic phase. Thus, the more acid present in protonated form, the greater the decrease in overall acid concentration in the aqueous phase and increase in the organic solvent. The total amount of acid in the aqueous phase is further determined by the volumetric ratio of aqueous and organic phase. These interactions influence the enzyme kinetics in a cumulative manner and mask the intrinsic pH-dependency of the enzyme.

To simplify the determination of pH-dependency of lipases during esterification as much as possible, investigations were performed in a biphasic system similar to a Lewis-cell (Figure 1), which is known from studies on mass transfer in multi-phase enzyme reactions (Bauer et al. 2002; Gargouri & Legoy, 1997). In this reactor, both liquid phases were agitated to exclude concentration gradients, while the interfacial area between the two layers was kept constant. The enzyme concentration was chosen in a way that a maximum conversion of 15 % occurred within 10 h. Within this period of time, almost no pH shift was detectable for low pH values while at higher values (pH > 6.25) a maximum pH shift of only 0.2–0.4 units was observed in preliminary experiments (data not shown). As spontaneous esterifications can occur especially under strongly acidic or basic conditions, the velocity of the autocatalysed reaction was determined. The maximum contribution of spontaneous esterification of butanol and propionic acid in the considered pH-range was found to be only in the order of 1–2% of the lipase-catalysed reaction (pH 3.5) and was therefore negligible.

It was found with the employed agitation regime of the reactor, that the mass transfer of butanol and propionic acid between aqueous and organic phase reached equilibrium after 30 min. As the time period over which the enzyme reaction was analysed was significantly longer (10 h), the mass transfer of reactants was of relatively minor importance and was therefore neglected.

At phase equilibrium (without enzyme present in the system), 17 % of the initial butanol remained in the hexane phase, independent of the pH of the aqueous phase. At pH values greater than 5, propionic acid was hardly detectable in the hexane phase due to the higher degree of propionic acid dissociation. In the case of an initial concentration of propionic acid of 300 mmol L^{-1}, at pH 2.75, 3% of the acid was detected in the hexane phase after equilibration.

The system characteristics described indicate that with the experimental setup employed and the chosen, well-defined conditions reliable results can be obtained.

pH-optima of lipase-catalysed esterifications

Esterification activities of CRL and TLL, revealed extremely low pH-optima of 3.5 and 4.25, respectively, at varying acid concentrations, as illustrated in Figure 2. The pH-optima are in the same range when the measured data are corrected to describe activities at constant 'effective' concentrations of protonated acid (Figure 2, dashed lines) and thus the intrinsic pH-dependency. This extrapolation was done by determination of mathematical correlations describing the dependency of the measured activity on the concentration of the protonated acid for each applied pH as illustrated in Figure 3. Assuming that this correlation is linear over a wide range, concentrations of propionic acid could also be calculated for higher pH values. (The underlying assumption that the protonated acid is a substrate for lipase catalysed esterification will be outlined in detail in the following section.) No substrate saturation or inhibition was observed within the applied concentration range, which was also verified in an experiment at

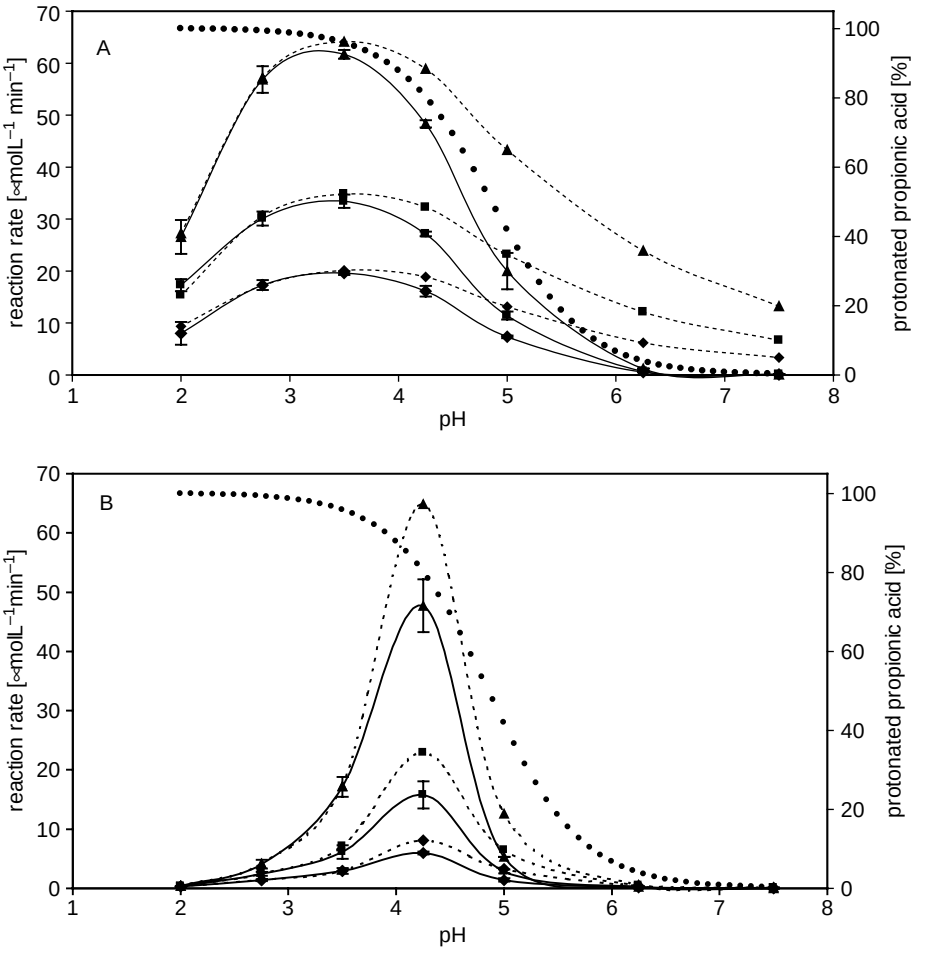

Figure 2. pH-optima for synthesis of butyl propionate at different initial concentrations of propionic acid catalysed by lipase of (A) CRL and (B) TLL. Black lines represent measured optima, dashed lines are extrapolated optima for constant concentrations of protonated propionic acid (▲ 300 mM; ■ 150 mM; ◆ 75 mM). The titration curve of propionic acid is represented by the dotted line.

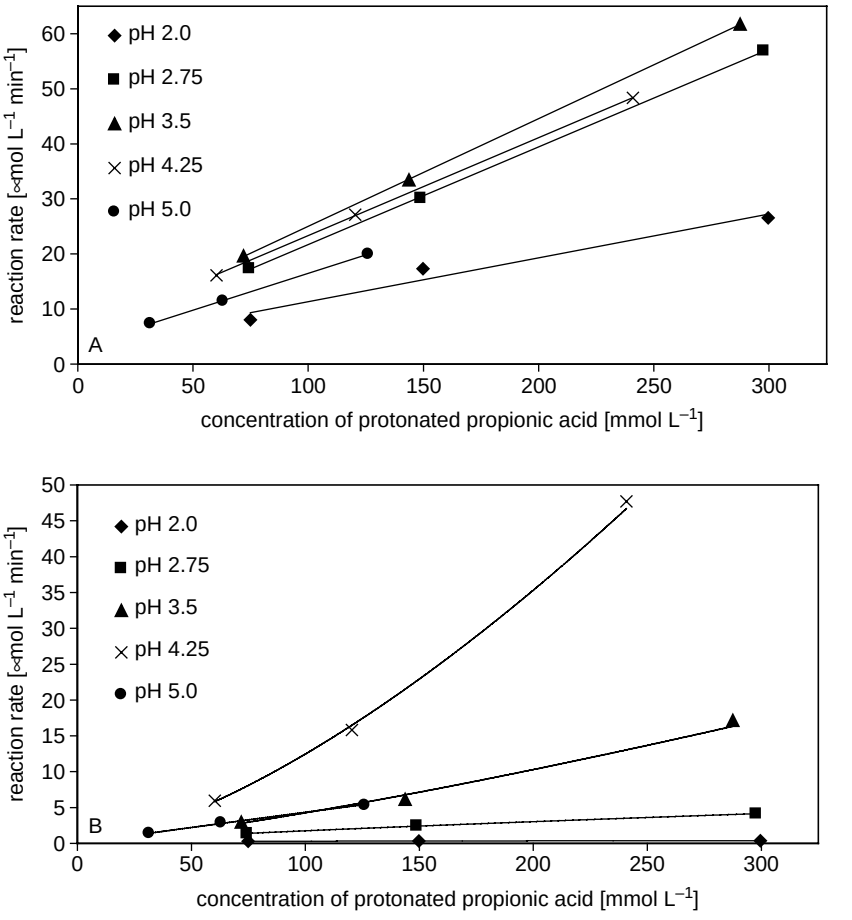

Figure 3. Activity (reaction rate) of CRL (A) and TLL (B) as a function of the concentration of protonated propionic acid at different pH values. Each regression line except for pH 2.0 (correlation coefficient appr. 0.95%) gave correlation coefficients of more than 0.99% for both enzymes. In case of TLL regression analysis revealed an exponential function for all pH values except for pH 2.0 and 2.75 where a linear equation was used.

the optimal pH of both enzymes up to total propionic acid concentrations of 2500 mmol L^{-1} for TLL and 3000 mmol L^{-1} (Figure 4).

While the pH-optima of both enzymes are very similar, the shape of the pH-dependency profiles is significantly different. For CRL, 80–90% of maximum activity was obtained within a range of ± 0.75 pH-units, while the activity of TLL drops to about 40% and 20% over the same pH-range. This difference might be ascribed to the presence of different isoforms of CRL with varying pH-optima in the commercial enzyme preparation (López et al. 2004). It is noticeable, however, that the intrinsic pH optimum of TLL is as narrow as the apparent one, while the intrinsic pH-optimum of CRL is extended to higher pH values. This indicates that the apparent pH-activity profile of CRL depends predominantly on the increasing concentration of protonated acid, whereas the activity of TLL responds to additional influences such as the protonation state of the biocatalyst itself.

The pH-optima found for esterifications are considerably lower than the known pH-optima of hydrolytic reactions (6.5–7.5 for CRL and 11–12 for TLL, Neves-Petersen et al. 2001). This is supported by the results of Crooks et al. (1995) who found optimum esterification of *n*-octanol and decanoic acid with lipases from *Humicola lanuginosa* and *Rhizomucor miehei* at a pH of 6.1, which was the lowest pH investigated. It is probable that further lowering the pH would have led to pH-optima in the range presented here. Astonishingly, to our knowledge a pH lower than 4.0 has so far never been checked in lipase catalysed esterifications. A reason for this might be the common perception that only enzymes from extremophiles can use low pHs. Additionally, at these pH values low stability is generally assumed. However, the stability of many lipases is not strongly affected by pH as was shown for CRL in a pH range of 3.0 to 7.0 by Montero et al. (1993). In fact, in the present study the measured reaction rate remained constant over the whole

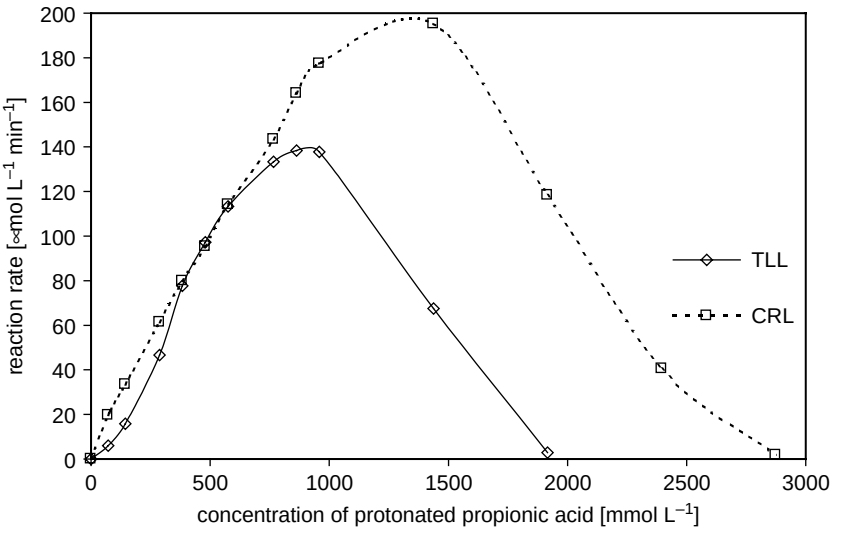

Figure 4. Influence of substrate concentration (non-dissociated propionic acid) on catalytic activity of CRL at pH 3.5 and TLL at pH 4.25.

reaction time, indicating that the lipases investigated were stable at low pH values.

Phenomena underlying pH-optima in esterification

The observed pH-dependency of both lipases implies that the dissociation state of propionic acid might play an important role for optimal activity. As lipase activity increases with increasing concentration of protonated acid, this protonated acid is probably the substrate for esterification. The coherence of this conclusion is evident from Figure 2 were the pH-dependent profiles of enzyme activity are correlated with the fraction of protonated acid. Especially for CRL, these profiles show a remarkably good agreement, i.e. the highest reaction rates are obtained when the concentration of the protonated acid is at 96% of the maximum. Optimal esterification activity of TLL can also be observed at very high concentrations of protonated acid, around 80–85% of maximum. The latter value indicates that, at least in the case of TLL, activity must be influenced by additional effects, apart from the availability of protonated acid. Otherwise, an absolute correlation of activity and protonated acid concentration would have occurred.

The influence of protonated acid concentration on enzyme activity can be understood by taking into account the investigation of lipase-catalysed hydrolysis on a molecular level, performed by Neves-Petersen et al. (2001). In that study the authors found the highest activity in pH ranges where the active site of the biocatalyst exposes a negative potential (neutral or alkaline pH). Due to this potential, fatty acids emerging from the cleavage of esters, fats and oils are spontaneously deprotonated, and finally ejected from the active site as a consequence of electrostatic repulsion. The corollary means that at neutral or alkaline pH a deprotonated, negatively charged acid cannot enter the active site and thus cannot be a substrate for lipase-catalysed esterification. According to Neves-Petersen et al. (2001) negative net charge can be found in the active site of CRL above pH 3.0, while TLL is negatively charged only above pH 8.0. Thus, while the net charge of CRL strongly supports the finding of a pH optimum as low as 3.5, the net charge of TLL would allow the deprotonated acid to enter up to pH 8.0.

The mechanism of catalysis in the active site of lipases also has to be taken into account. The nucleophilic hydroxy group of the serine residue in the catalytic triad is not capable to efficiently attacking the carbonyl-C-atom of a deprotonated acid with its delocalised negative charge. Thus, only at pH values below the pKs of the acid can lipase-catalysed esterification be performed. This would be true, even if the enzyme is in a more favourable conformational state at higher pH values. In addition to the influence on the protonation state of the acid, a low pH might alter the reaction mechanism at the catalytic triad of the biocatalyst, e.g. by protonation of the Asp (Glu) residue, as speculated by Neves-Petersen et al. (2001) and Paiva et al. (2000). This can neither be concluded nor excluded from the results presented here, and thus must await further investigations.

Nevertheless, esterfication at a pH, where high concentrations of protonated acid are present is feasible. Figure 4 demonstrates that this acid concentration can be increased to 1000 mmol L^{-1} for CRL and 600 mmol L^{-1} for TLL at the optimum pH without substrate saturation or inhibition. Concentrations higher than 1000 mmol L^{-1} or 1500

mmol L^{-1} lead to a rapid inactivation (Figure 4), which cannot be correlated with common inhibition terms. This is a remarkable result, especially since Krishna and Karanth (2001) reported a typical asymptotic course of lipase activity in a comparable investigation. Sigmoid kinetic behaviour of TLL, might be explained by the so-called slow-transition model, also known as kinetic co-operativeness (Ainslie et al. 1972), or by formation of associated enzyme complexes. Nini et al. (2001) observed sigmoidal kinetics of TLL when investigating interfacial activation in the hydrolysis reaction, but without giving an explanation. The underlying mechanism would be an interesting subject for further investigation.

Conclusion

The results presented rebut the general assumption that pH-optima of lipase-catalysed esterification is similar to that of hydrolysis. For penicillin acylase, a hydrolase with a different catalytic mechanism, this was reported a long time ago (Kaufmann & Bauer 1960), but this has not been established with lipase catalysis. Based on the finding that the protonated acid is probably used as the substrate, it may be assumed that the optimal pH for most lipase-catalysed esterifications will be below the pKs of the organic acid employed. This might even be valid for all hydrolases employing a comparable catalytic mechanism. The good performance of the biphasic reaction systems most frequently applied for esterifications can only be explained by failure of the buffer systems employed producing a favourably low pH within the aqueous phase. The findings reported in this study will facilitate a more rational application of lipases to esterifications.

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